BEHAVIORAL RESPONSES TO DDT AND PYRETHROIDS BETWEEN *ANOPHELES MINIMUS* SPECIES A AND C, MALARIA VECTORS IN THAILAND

JINNAPA POTIKASIKORN, THEERAPHAP CHAREONVIRIYAPHAP,* MICHAEL J. BANGS,
AND ATCHARIYA PRABARIPAI

Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand; United States Naval Medical Research Center, Silver Spring, Maryland; Division of Computer and Biostatistics, Faculty of Liberal Arts and Sciences, Kasetsart University, Nakhonpathom, Thailand

Abstract. Behavioral responses of two field populations of *Anopheles minimus* complex species A and C for contact and non-contact actions of chemicals were compared during and after exposure to operational field concentrations of DDT (2 g/m^2), deltamethrin (0.02 g/m^2), and lambda-cyhalothrin (0.03 g/m^2) using an excito-repellency escape chamber. The two populations were collected from the Mae Sot District in Tak Province (species A) and the Tri Yok District in Kanchanaburi Province (species C) in western Thailand. Female mosquitoes of both populations rapidly escaped from chambers after direct contact with DDT, deltamethrin, and lambda-cyhalothrin. The non-contact repellency response to DDT and the two synthetic pyrethroids was pronounced with *An. minimus* species A; however, non-contact repellency was relatively weak with *An. minimus* species C, but remained significantly greater than the paired controls ($P < 0.05$). We conclude that strong contact irritancy was present in both test populations, whereas non-contact repellency also played a significant role in the escape response of *An. minimus* species A.

INTRODUCTION

Anopheles minimus s.l. Theobald is one of the most efficient malaria vectors throughout the eastern Asia.[1,2] In Thailand, the *An. minimus* complex contains important vectors of malaria that are found exclusively in the forested hilly and clear forested foothill areas.[3–6] *Anopheles minimus* s.l. was reported to be mostly endophilic and endophagic throughout its geographic range.[7] After DDT was introduced to interrupt malarial transmission, *An. minimus* reportedly shifted to greater outdoor feeding and more zoophilic preferences for blood, particularly bovids.[8,9] Even though DDT resulted in significant reductions of indoor-feeding mosquitoes, this control method did not completely interrupt transmission of malaria. This has been attributed partly to exophagic behavior of portions of the population and the persistence of a small number of vectors that enter and successfully feed indoors.[10,11] Similar observations have also reported from Vietnam,[12] raising questions on behavioral variations within the *An. minimus* taxon.

Based on morphologic and genetic variations, at least two closely related species of the *An. minimus* complex have been documented in Thailand and both have been incriminated as efficient vectors of malaria.[4,6] *Anopheles minimus* species A is the predominant species and distributed throughout the country,[6] whereas species C appears restricted along the western Thailand-Myanmar border, particularly in Kanchanaburi Province.[4,13] Additionally, *An. minimus* species D has been reported in Thailand, but sufficient information is lacking to support the proposed sibling species status.[13] Although *An. minimus* species A and C occur in sympatry in western Thailand, notable ecoethologic variation in feeding and resting behaviors, degree of anthropophily, and other bionomical aspects may influence vector capacities of these two sibling species.[12,14]

Anopheles minimus species A has shown a much greater (five-fold difference) endophilic behavior compared with species C.[12] The *An. minimus* complex has also shown different response levels of response to intradomiciliary use of insecticides.[15–19] In Thailand, indoor house spray has been routinely conducted to interrupt human-vector contact and transmission.[19] Understanding the behavioral responses of different species of mosquitoes, even closely related sibling species, to insecticides can facilitate vector control by selecting and implementing the most effective interventions possible and help to target the primary disease vectors.

Behavioral responses, namely insecticide avoidance, can be separated into two important and distinct categories: contact irritancy and non-contact repellency. Irritant responses result from physical contact with chemical-treated surfaces, whereas repellency is an avoidance response devoid of making actual contact with insecticides.[20] Although behavioral responses have been recorded with various mosquito species and populations of *Anopheles* from Thailand using the excito-repellency test box,[19,21–23] none have been recorded to compare the behavioral responses between species in the *An. minimus* complex (e.g., species A and C). Described herein are observations using the excito-repellency test system to quantitatively measure behavioral responses between wild-caught populations of *An. minimus* species A and C exposed to recommended field concentrations of DDT, deltamethrin, and lambda-cyhalothrin.[24]

MATERIALS AND METHODS

Mosquito collection. *Anopheles minimus* complex mosquitoes were identified based on morphologic keys.[15,25] Species were differentiated by the presence or absence of the humeral pale spot on the costal wing vein. *Anopheles minimus* A has a wing costa without the humeral pale spot whereas *An. minimus* C has the humeral pale spot. A diagnostic enzyme, octanal dehydrogenase, indicated 95% concurrence with species A, which does not have the humeral pale spot. This spot is lacking in 73% of species C.[6] *Anopheles minimus* A and C adult females were collected off human volunteer baits during the evening hours (6:00 PM to 6:00 AM). These volunteers (collectors) worked for the Ministry of Public Health. Behavioral tests were performed within 24 hours of capture. All

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mosquitoes were starved of blood and sugar 24 hours before the tests.[22] Temperatures and relative humidity were recorded during the tests. Both populations were physiologically susceptible to DDT, deltamethrin, and lambda-cyhalothrin (Chareonviriyaphap T and others, unpublished data).

Insecticide-treated papers. Analytical grade insecticide was impregnated on papers at operational field concentrations of 2 g/m^2 of DDT, 0.02 g/m^2 of deltamethrin, and 0.03 g/m^2 of lambda-cyhalothrin and prepared using diluent according to World Health Organization protocol.[26]

Behavioral tests. Tests were designed to compare two wild-caught populations in contact versus non-contact exposures using three different insecticides. Identical, specially designed test chambers (four per test trial) were used for all bioassays as previously described.[27] The stainless steel outer chamber of excito-repellency testing device measures 34 cm × 32 cm × 32 cm (Figure 1), and faces the front panel with the single escape portal. The box is composed of a rear door cover, an inner Plexiglas glass panel with a rubber latex-sealed door, a Plexiglas holding frame, a screened inner chamber, an outer chamber, a front door, and an exit portal slot. Only female *An. minimus* specimens were used in excito-repellency tests. Mosquitoes were deprived of all nutrition and water for a minimum of 24 hours before exposure. Laboratory tests were performed during daylight hours only and each test was replicated four times. Observations were taken at one-minute intervals for 30 minutes. After each test was completed, the number of dead or knockdown specimens was recorded separately for each exposure chamber, external holding cage, and paired control chamber (without insecticide). Escaped specimens and those remaining inside the chamber, for both controls and treatments, were held separately in small holding containers with food and water and 24-hour mortalities were recorded.

Data analysis. A Kaplan-Meier survival analysis method was used to analyze and interpret the behavioral response data.[20–22,27] Survival analysis was used to estimate the probability of escape time (ET) and compare differences in mosquito response among the two populations and three insecticides. Mosquitoes that escape were treated as deaths and those remaining in the test chamber were considered survivals.[21] The ET$_{50}$, ET$_{75}$ and ET$_{90}$ time in minutes for 50%,

75%, and 90% of the test population to escape, respectively, were estimated from data collected at one-minute intervals. Patterns of escape response were determined using the log-rank method.[28] Stata statistical software (Stata Corp., College Station, TX) was used in the analysis.[20]

RESULTS

Two types of behavioral responses, contact irritancy and non-contact repellency, were observed with exposure to three insecticides and percent mortalities of escape and non-escape mosquitoes from control and treated chambers were recorded (Table 1). Patterns and rate of escape were stronger in *An. minimus* species A than *An. minimus* species C for all three compounds. In contact trials, percent escape of *An. minimus* A (92–96%) was significantly ($P < 0.05$) higher than for *An. minimus* C (50–90%), regardless of compound used. Similarly, percent escape by species A was also greater than that by species C for the two synthetic pyrethroids. In general, a relatively low number of mosquitoes escaped from the control chambers (12–23% for contact and 10–15% for non-contact). Mortality rates of escaped mosquitoes from both test populations were low (0–13.3%), whereas those that remained in the test chamber (non-escape mosquitoes) showed much higher mortality rates (43–100%). All non-escape specimens of species A exposed to deltamethrin and lambda-cyhalothrin perished within 24 hours post-exposure (Table 1). High mortality rates (13.3%) of escaped mosquitoes from control chambers were observed with DDT. In non-contact trials, *An. minimus* species A demonstrated significantly strong escape

FIGURE 1.   Excito-repellency test chamber used to study insecticide behavioral responses.

7. Exit portal
6. Front door
5. Outer chamber
4. Screened inner chamber
3. Plexiglass holding frame
2. Plexiglass panel with rubbered door
1. Rear door cover

TABLE 1

Percentage escape response and mortality of *Anopheles minimus* A and C exposed to DDT, deltamethrin, and lambdacyhalothrin in contact and noncontact trials*

Condition	Population	Chemical (No. of tests)	Number escaped (%)	% mortality Escaped	% mortality Remained
Contact					
	Species A	DDT (85)	78 (92)	0	42.8
		DDT-C (85)	10 (12)	0	0
		Del (76)	73 (96)	0	100
		Del-C (75)	17 (23)	0	0
		Lam (77)	72 (94)	1.4	100
		Lam-C (78)	18 (23)	0	0
	Species C	DDT (100)	77 (77)	1.3	0
		DDT-C (100)	15 (15)	13.3	1.2
		Del (98)	50 (51)	2	0
		Del-C (94)	13 (14)	0	1.2
		Lam (100)	90 (90)	1.1	0
		Lam-C (100)	18 (18)	0	0
Non-contact					
	Species A	DDT (85)	82 (96)	0	0
		DDT-C (83)	22 (27)	0	0
		Del (76)	70 (92)	0	0
		Del-C (75)	22 (29)	4.5	0
		Lam (77)	67 (87)	1.5	0
		Lam-C (77)	21 (27)	0	0
	Species C	DDT (100)	24 (24)	0	0
		DDT-C (100)	10 (10)	0	0
		Del (100)	20 (24)	0	0
		Del-C (100)	10 (10)	0	1.1
		Lam (95)	17 (18)	0	0
		Lam-C (95)	14 (15)	0	0

* DDT = 2 g/m^2; deltamethrin (Del) = 0.02 g/m^2; lambda-cyhalothrin (Lam) = 0.03 g/m^2; C = control tests (without insecticides).
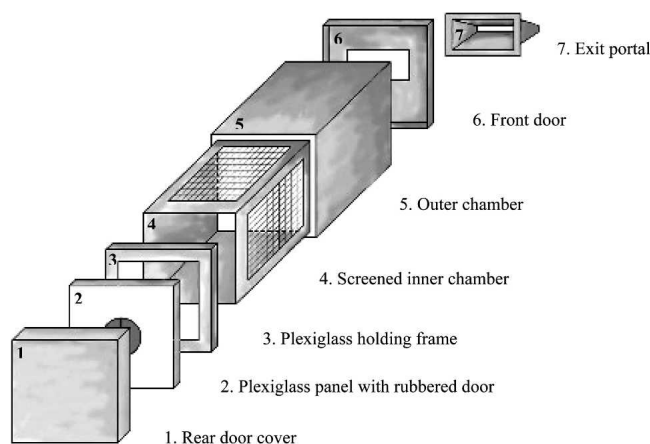
responses to all three compounds compared with species C. After 30 minutes exposure, percent escape was approximately 96% for DDT, 92% for deltamethrin, and 87% for lambda-cyhalothrin with *An. minimus* species A, while only 24% for DDT and deltamethrin and 18% for lambda-cyhalothrin with species C. Percent mortalities of escaped specimens of both populations were very low, ranging from 1.1% to 4.5%. Mortality was not seen in non-escaped *An. minimus* species A after the 24-hour holding period.

The escape patterns generated from insecticide-treated chambers are expressed in one-minute intervals for 50%, 75%, and 90% (ET_{50}, ET_{75}, and ET_{90}) of the test population to escape from exposure chambers (Table 2). In contact trials, the ET_{50}, ET_{75}, and ET_{90} for *An. minimus* species A were 5, 12.5, and 24 minutes with DDT, 2.5, 6, and 16 minutes with deltamethrin, and 7, 23.5, and 30 minutes with lambda-cyhalothrin, respectively. The ET_{50} for *An. minimus* C was 5, 8.5, and 6 minutes for DDT (0.02 g/m^2). The ET_{75} and ET_{90} values for all three compounds for *An. minimus* species C could not be calculated (with one exception: lambda-cyhalothrin ET_{75} = 12.5 minutes) because of insufficient numbers of mosquitoes escaping. Similarly, ET values for *An. minimus* species C in all non-contact trials could not be estimated. For non-contact trials, the ET_{50}, ET_{75}, and ET_{90} values were 5, 4.5, and 14 minutes for DDT and 5.6, 8, and 25 minutes for deltamethrin. The ET_{50} and ET_{75} estimates were 6.5 and 23.5 minutes, respectively, for lambda-cyhalothrin.

Comparison of escape responses between *An. minimus* species A and C in contact and non-contact trials using log rank analysis showed statistically significant differences in escape patterns between species in non-contact trials for all three compounds ($P < 0.05$). In contact trials, significant differences in escape responses between species were observed with DDT and deltamethrin ($P < 0.05$). Comparisons of escape responses between paired contact and control, contact and non-contact, and non-contact and control bioassays for each species for each compound were made. No significant differences between contact and non-contact escape for *An. minimus* species A were observed ($P > 0.05$). Escape probabilities in contact and non-contact trials were significantly higher than those in paired controls for all bioassays.

Figures 2–5 show the proportions of mosquitoes remaining in the exposure and control chambers under different test conditions and chemical exposure. Strong repellency action was seen with *An. minimus* species A against all three compounds, whereas significantly less escape reaction was observed with *An. minimus* species C (Figure 5). In non-contact tests, *An. minimus* species A demonstrated a stronger escape rate with DDT than with either deltamethrin or lambda-cyhalothrin (Figure 5). There were significant differences in escape responses seen in all contact trials compared with paired control and non-contact trials with *An. minimus* species C ($P < 0.05$). Escape patterns in all non-contact repellency trials for *An. minimus* species A were significantly greater than paired controls.

DISCUSSION

In Thailand, *An. minimus* complex is comprised of at least two known species, species A and C, and both are important vectors of malaria in Thailand.[3,4,6,15] After DDT was introduced for malaria control in 1949, *An. minimus* reportedly became predominately an outdoor-feeding species,[5] although it appears that feeding behavior varies depending upon geographic distribution. Thus, insecticides may have little to do with any purported genetic selection or shift from an indoor to outdoor-feeding behavior.[10] The failure to completely interrupt malarial transmission by *An. minimus* s.l. might be related to the behavioral diversity and innate response to insecticidal intervention.[5,29]

Studies have attempted to quantitatively describe and resolve the ecoethologic differences,[9,10,12,14,30] genetic composition and diversity,[4,6,12,30–33] and responses to intradomicilary use of DDT[30] in this species complex. Experiments using recently colonized *An. minimus* species A exposed to deltamethrin clearly demonstrated the two primary avoidance responses: irritancy and repellency (excito-repellency).[19] In our present study, we compared both behavioral responses in the two sibling species of *An. minimus* present in Thailand to three different residual insecticides used in public health with hopes that such information will facilitate targeting of specific malaria vectors and increase the effectiveness of vector control activities.

We observed unambiguous behavioral avoidance responses in *An. minimus* species A and C using an excito-repellency test system.[27] All three insecticides produced rapid and striking irritancy in both sibling species. Moreover, very strong repellency responses to each compound were observed in *An. minimus* species A. Repellency reactions were similar to those of a recent laboratory colony of *An. minimus* species A from northern Thailand, which showed > 75% repellency to deltamethrin.[19] Repellency responses were relatively weak in *An. minimus* species C, yet still significantly greater than the paired controls for all cases. Similarly, weak repellency of *An. minimus* species C from Pu Teuy Village (approximately 95% were confirmed as species C) to the three compounds was previously observed.[19] *Anopheles minimus* complex from Pu Tuey village in Kanchanaburi Province was exposed to operationally standard concentrations of DDT (2 g/m^2) and established medium lethal doses (LD_{50}) of deltamethrin and lambda-cyhalothrin that produced poor repellency activity.[19] The relative inability to detect chemical signals or odors without physical contact with insecticide in *An. minimus* species C may be driven by evolutionary processes different from those in species A. Since 1990, Pu Teuy village has been considered a low-risk area for malaria, which has resulted in routine residual chemicals being applied more sparingly compared with more malaria-prone areas of the country such as Mae Sot

TABLE 2
Escape time (ET) in minutes for 50%, 75%, and 90% of *Anopheles minimus* A and C to escape from insecticide-treated chambers*

	DDT			Deltamethrin			Lambda-cyhalothrin		
	ET_{50}	ET_{75}	ET_{90}	ET_{50}	ET_{75}	ET_{90}	ET_{50}	ET_{75}	ET_{90}
Contact									
Species A	5	12.5	24	2.5	6	16	7	23.5	30
Species C	5	–	–	8.5	–	–	6	12.5	–
Non-contact									
Species A	2?	4.5	14	5.6	8	25	6.5	23.5	–
Species C	–	–	–	–	–	–	–	–	–

* Survival analysis was used to estimate time in minutes for 50%, 75%, and 90% of test populations to exit chambers. DDT = 2 g/m^2; deltamethrin = 0.02 g/m^2; lambda-cyhalothrin = 0.03 g/m^2.
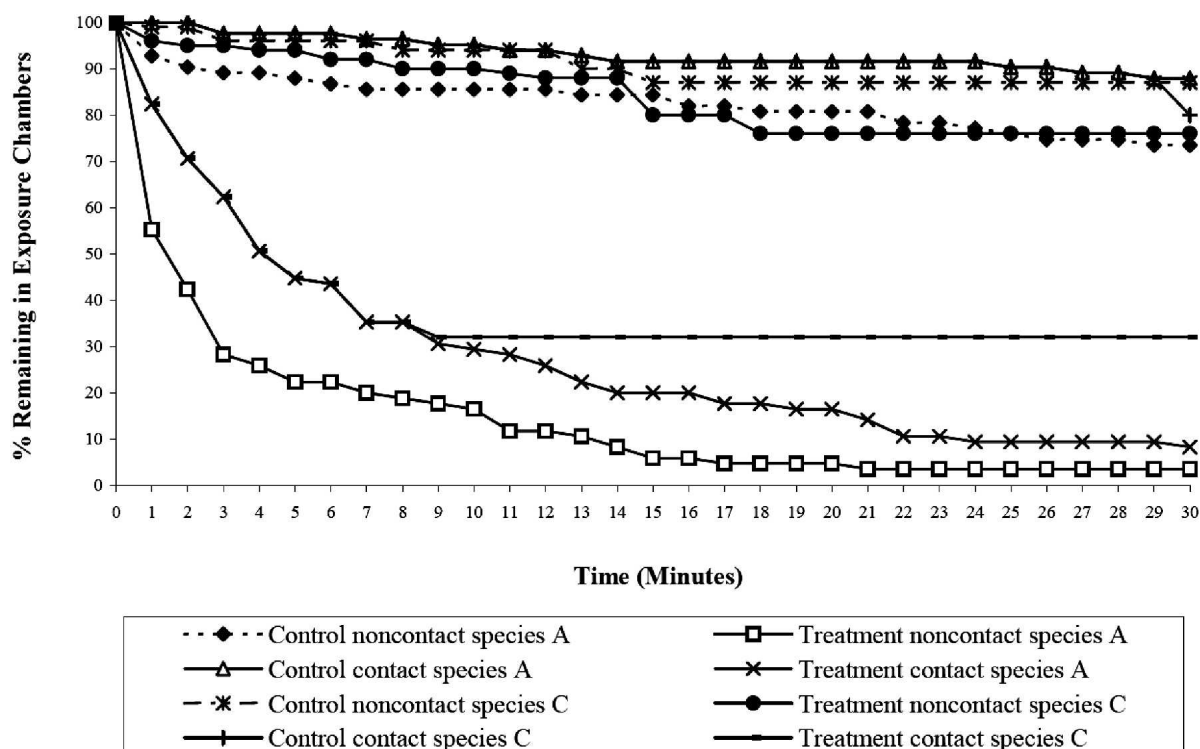
FIGURE 2. Escape probability of *Anopheles minimus* species A and C exposed to DDT and paired control chambers for contact and non-contact trials.

District (Department of Communicable Disease Control, 2004, unpublished data). The differences in proportion of total houses sprayed with insecticides (i.e., insecticide exposure pressure) could be a factor affecting the avoidance behavior of these two closely related species.

One of the key components in preventing malaria transmission has relied mainly on methods that interrupt human-vector contact.[34–36] Insecticides that have strong irritant and repellency attributes on vectors can perform this function without necessarily having to kill the mosquito to interrupt
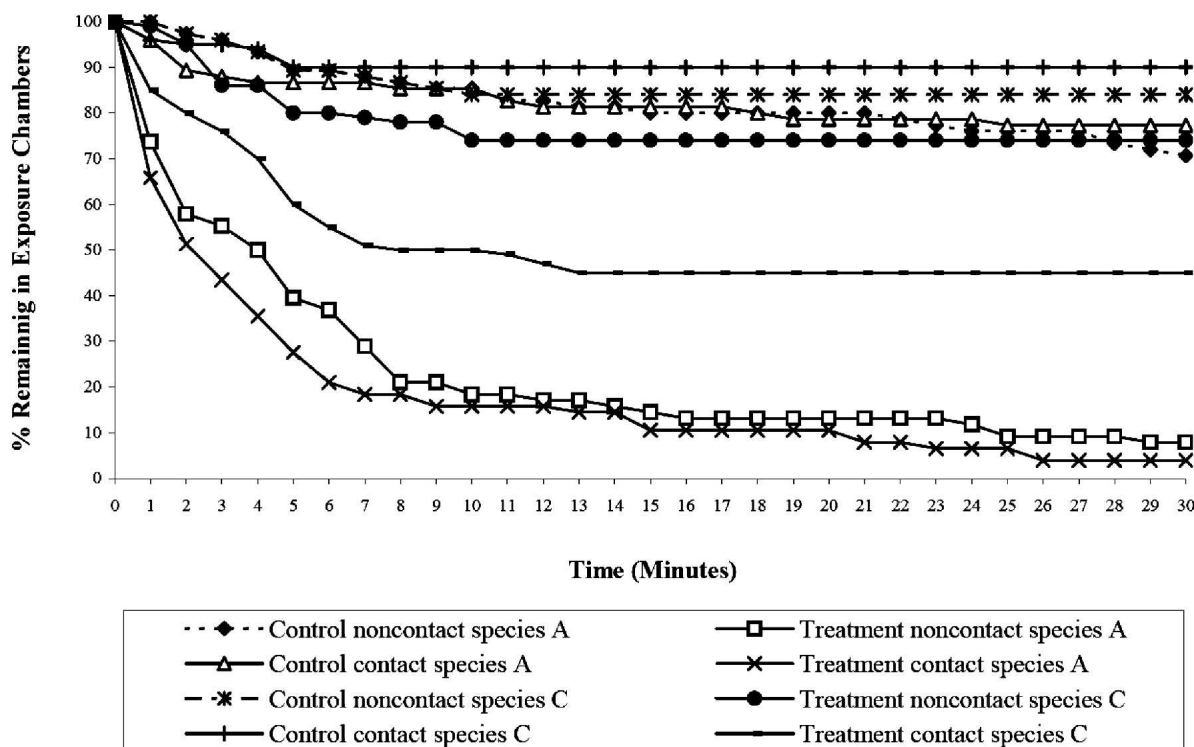

FIGURE 3. Escape probability of *Anopheles minimus* species A and C exposed to deltamethrin and paired control chambers for contact and non-contact trials.
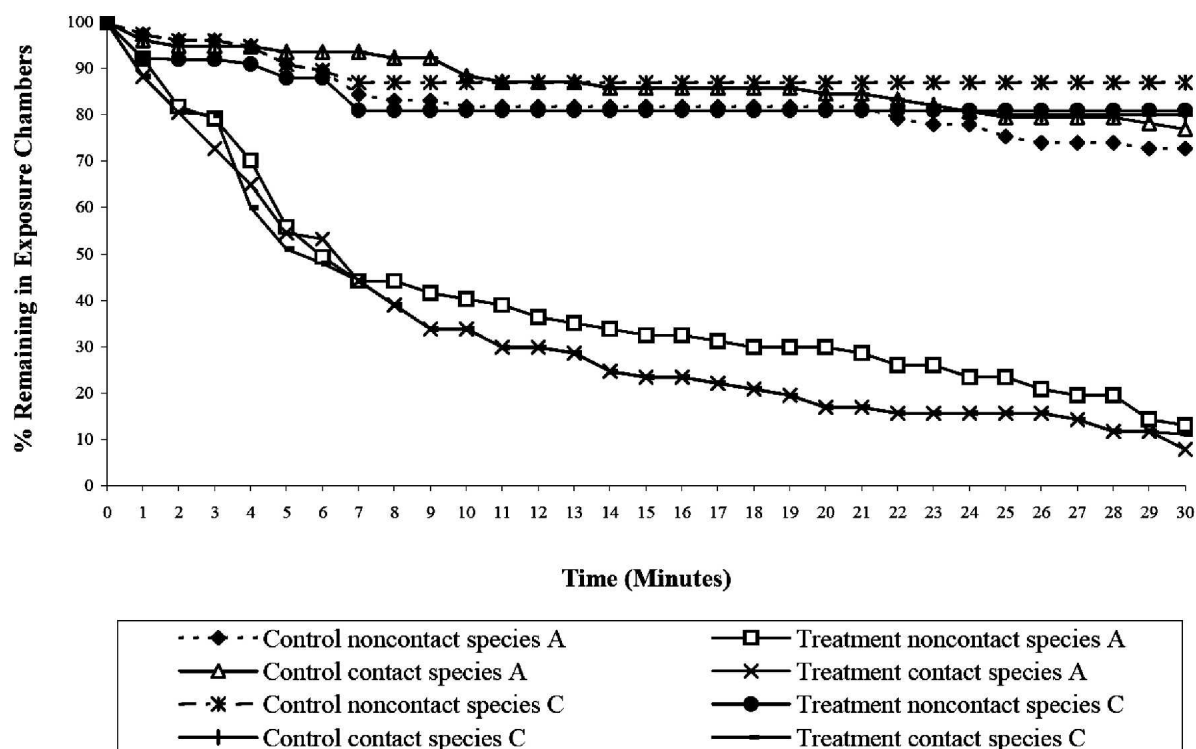
FIGURE 4.   Escape probability of *Anopheles minimus* species A and C exposed to lambda-cyhalothrin and paired control chambers for contact and non-contact trials.

transmission. Repellency to insecticides in vectors has been recognized in several *Anopheles* mosquitoes.[17,18,21,22,23,36–38] Compared with contact irritancy, this type of avoidance behavior could mitigate even more against selection of insecticide resistance in mosquito populations.

Anopheles minimus species A in Thailand, has been subjected to routine intradomicilary DDT spraying to interrupt malaria transmission for decades. DDT was applied either once or twice a year, especially in malaria-endemic areas of western Thailand. Although DDT was used for many years,
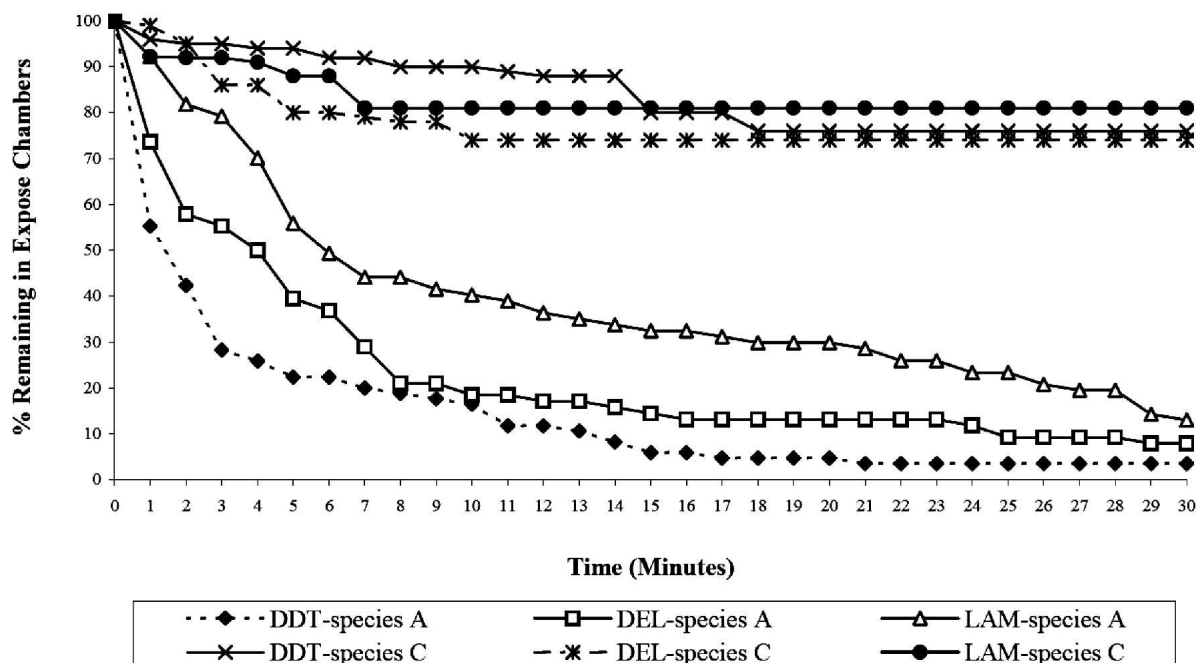

FIGURE 5.   Escape probability of *Anopheles minimus* species A and C exposed to DDT, deltamenthrin (DEL), and lambda-cyhalothrin (LAM) in non-contact trials.

no evidence of physiologic resistance has been detected in the *An. minimus* complex. We believe that innate behavioral avoidance of insecticide-sprayed surfaces by mosquitoes has, and continues to play, a significant role in delaying or preventing resistance from developing. Our findings confirm that strong behavioral avoidance of chemical residues is due to excito-repellent properties of these compounds and most likely contributes to interruption of feeding by mosquitoes and transmission of malaria.

Our findings indicate differences in behavioral responses between two species of the *An. minimus* complex in Thailand. We believe that these important observations can help explain some of the varying effectiveness of indoor residual spraying in various regions in Thailand. It is the understanding of behavioral avoidance and an appreciation for excito-repellency that indicate an important set of properties of residual insecticides and how they function to control disease transmission apart from contact toxicity alone.

Authors' addresses: Jinnapa Pothikasikorn and Theeraphap Chareonviriyaphap, Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand. Michael J. Bangs, Naval Medical Research Center, 503 Robert Grant Avenue, Room 3A40, Silver Spring, MD 20910-7500. Atchariya Parbaripai, Division of Computer and Biostatistics, Faculty of Liberal Arts and Science, Kasetsart University, Nakhonpathom 73140 Thailand.

Reprint requests: Theeraphap Chareonviriyaphap, Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand, Telephone: 66-2-942-7131, Fax: 66-2-942-7130, E-mail: faasthc@ku.ac.th.

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